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following conclusions were drawn: (1) The light chain of the nicked (dichain) type B is derived from the N-terminal one third of the single chain (unnicked) parent neurotoxin; (2) sequence homologies are present between single chain types B and E and the light chain of the nicked type A (Schmidt, J. J., Sathyamoorthy, V., and DasGupta, B. R., 1984, Biochem. Biophys. Res. Comm. 119, 900-904; (3) the N-terminal regions of the heavy chains of types A and B have some structural similarity; (4) activation of type B neurotoxin cannot involve removal of amino acids or peptides from the N-terminus.

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Partial Amino Acid Sequences of Botulinum Neurotoxins Types B and E

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Running Title: Structures of Botulinum Neurotoxins Types B and E

Subject Category: Protein Structure, Function, and Turnover

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ABSTRACT

Clostridium botulinum type E neurotoxin, a single chain protein of M_r 147,000, was purified and subjected to amino acid sequencing. The same was done for single chain botulinum type B neurotoxin (M_r 152,000), and for the heavy and light chains (M_r 104,000 and 51,000 respectively) derived from type B by limited trypsin digestion. Twelve to 18 residues were identified and the following conclusions were drawn: (1) The light chain of the nicked (dichain) type B is derived from the N-terminal one third of the single chain (unnicked) parent neurotoxin; (2) sequence homologies are present between single chain types B and E and the light chain of the nicked type A (Schmidt, J. J., Sathyamoorthy, V., and DasGupta, B. R., 1984, Biochem. Biophys. Res. Comm. 119, 900-904); (3) the N-terminal regions of the heavy chains of types A and B have some structural similarity; (4) activation of type B neurotoxin cannot involve removal of amino acids or peptides from the N-terminus.

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INTRODUCTION

The singular cause for the neuroparalytic disease botulism is a simple protein. The anaerobic bacterium Clostridium botulinum produces neurotoxin in seven serologically distinguishable types (A through G). Each NT³ type is synthesized as a single chain polypeptide. Depending on the physiology of the bacteria the single chain protein ($M_r \sim 150,000$) may be cleaved ("nicked") endogenously in the bacterial culture to a dichain protein. The two chains, called heavy ($M_r \sim 100,000$) and light ($M_r \sim 50,000$), of the dichain NT are held together by disulfide bond(s) (1). Therefore, the NT may be found in the bacterial cultures as a single chain (e.g. type E), a dichain (type A), or a mixture of single and dichain (type B) proteins. The dichain (nicked) neurotoxins produced naturally, i.e. in the bacterial culture, or produced artificially, i.e. with trypsin, are electrophoretically indistinguishable (2).

Pharmacological (3) and other studies (4,5) suggest that the heavy chain of the NT binds to the cholinergic presynaptic membrane and then part of the NT (presumably the light chain) causes neurotoxicity on entering the cholinergic nerve ends by blocking the depolarization-evoked release of acetylcholine. This tentative structure-function relationship for the chains of botulinum NT is somewhat similar to those of diphtheria, Pseudomonas, and cholera toxins, and ricin and abrin (5,6), in that each of these toxic proteins consists of two polypeptide chains. One is responsible for binding with the target tissue and the other for toxic activity. In view of this general common structure-function relationship, biochemical characterization of the heavy and light chains of botulinum NT is of obvious importance.

Here we report the first partial characterization of type B NT based on sequence studies of the single chain molecule, the two chains from the dichain (nicked) molecule, and also the partial amino acid sequence of single chain type E toxin. This information provides a chemical basis to compare the immunologically distinct but pharmacologically similar botulinum NT types A, B, and E.

MATERIALS AND METHODS

The single chain type E NT was purified (7) from cultures of C. botulinum type E (strain Alaska E-43, kindly supplied by Dr. Lynn Siegel, Fort Detrick, Frederick, MD). The NT, M_r 147,000 (8), appeared as a single band (Coomassie Brilliant Blue staining) in 5% polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate, as seen before (7). Type B NT, M_r 152,000 (8), was purified (9) from C. botulinum type B (strain Okra). To convert single chain type B NT to the dichain form, the preparation was digested with trypsin (EC3.4.4.4., tosylamido-2-phenylethyl chloromethylketone treated, Worthington Biochemicals Lot #32A680); NT:enzyme ratio 40:1 w/w, at 37°C in 0.1 M Na acetate buffer pH 6.0, 30 min. Reaction was terminated with the protease inhibitor, phenylmethylsulfonyl fluoride (Sigma). Complete conversion of the single chain to the dichain form was avoided to minimize or prevent reported secondary fragmentations in addition to the nicking (10). The trypsinized NT, now mostly in the dichain form, was reduced with dithiothreitol to break the disulfide bond(s) holding the heavy and light chains together. These two chains were separated and purified by adapting the ion exchange chromatographic methods that were used for the separation and purification of the heavy and light chains of type C and type A NT (11,12). Details of these modifications will be published elsewhere. The isolated light and heavy chains (M_r 51,000 and 104,000, respectively, see ref. 8) of type B each migrated as a single band in 5% polyacrylamide gel electrophoresis containing 0.1% sodium dodecylsulfate.

The protein samples were carboxamidomethylated as described (13) and then subjected to automated Edman degradation in a Beckman 890C sequencer (13). The PTH amino acids were identified and quantitated by HPLC (Waters Associates, Milford, MA) using the procedure of Henderson et al. (14) with

minor modifications. Amino acid assignments were made by comparing chromatograms of each cycle to that of a PTH standard mixture, and also to the chromatogram of the preceding cycle. Identification criteria required that one residue show at least a twofold increase in amount compared to the preceding cycle, and no other show more than a 1.2-fold increase.

RESULTS

Figure 1 shows electrophoresis results on purified preparations of botulinum NT types B and E, on trypsin-treated (nicked) type B, and on the individually purified preparations of the heavy and light chains from nicked type B. As usual, type E was found to be non-nicked and migrated as a single band in the presence of detergent and reductant. Preparations of type B (2) used in these experiments also proved to be essentially the single chain form with but a trace of dichain, and thus were directly suitable for sequence studies.

Figure 2 shows representative HPLC chromatograms of the first cycles from sequencer runs on single chain types E and B, and on the heavy and light chains derived from nicked type B and then purified. Proline is the N-terminal residue of both single chain neurotoxins and of the light chain of type B, while alanine is the N-terminus of type B heavy chain. The chromatograms attest to the purity of the preparations and to the likelihood that each individually purified protein consists of a single polypeptide chain. The latter observation is of particular importance for reasons presented in the discussion section.

Sequence results on the four proteins are summarized in Figure 3. Identical sequence data were obtained from duplicate analyses of each sample. For the duplicate analyses of single chain (non-nicked) protein (type B or E) the NT was purified from two separately produced cultures. Similarly, the heavy and light chains of type B were also isolated from two separately purified batches of type B. Compared to replicate analyses of a single batch of NT and its component chains, analyses of two separately prepared batches allowed a more rigorous examination of the integrity of the N-terminals and the sequence of several amino acids.

The structures of the N-terminal regions of types B, E, and the light chain of type A (13) are compared in Figure 4a, while Figure 4b shows a comparison of the heavy chains from types A and B. In both figures, significant homology is demonstrated.

DISCUSSION

The N-terminal sequences of the single chain (non-nicked) type B and the light chain purified from nicked type B are identical. This proves that the light chain is the amino terminal end of the original single chain prior to nicking and that the site of nicking is about one third the distance from the N- to C-terminal, an arrangement heretofore predicated solely on an assumed analogy with the situation for tetanus toxin. Further, it is clear that the heavy chain (M_r 104,000) is not a dimer of the light chain (M_r 51,000) and that the heavy chain is not a heteropolymer of two polypeptides that might appear from the cleavage of the single chain type B NT (M_r 152,000) at two sites (i.e., cleavage in addition to nicking). This possibility was entertained because of the conjecture that the NT is an aggregate of smaller subunits (15), and that some proteins might remain aggregated in sodium dodecylsulfate-polyacrylamide gel electrophoresis (ref. in 16); a good example is bovine serum albumin.

Alignment of the N-terminal regions of types B, E, and the light chain of type A, as shown in Figure 4a, reveals extensive sequence homology, the first such direct evidence for this situation among the various serotypes of botulinum neurotoxin. This comparison also suggests that, as directly demonstrated for type B NT, the light chain of type A NT probably represents the N-terminal region of single chain parent NT. Finally, it suggests that the same is likely to be true for the dichain form of type E (produced by limited proteolysis of single chain E with trypsin).

The heavy chains of types A (13) and B are structurally similar, as seen in Figure 4b, with eight of the first sixteen residues identical. Further, one of the differences involves the substitution of isoleucine for leucine, a very conservative replacement. Both chains as sequenced have

carboxamidomethyl cysteine near the N-terminus, but it has not yet been determined whether or not this residue is cysteine or half-cystine in the respective native protein.

These results also provide information with respect to certain aspects of activation and nicking. Following its synthesis, the relatively low toxicity of the single chain NT increases significantly after partial proteolysis by enzyme(s) endogenous to the bacteria. A dichain (nicked) NT, isolated from the culture, is generally regarded as fully activated. Trypsinization of the single chain NT results in its nicking and generally also in an increase in its specific toxicity (activation). However, a direct causal relationship between nicking and activation has not been established (1,2,10). Activation in the bacterial culture or by trypsin may involve cleavage of at least two bonds (1,2), of which cleavage at the site of nicking (one-third the distance from N- to C-terminal) is one. Another cleavage might occur but remain undetected by the techniques used so far (i.e. gel electrophoresis) if the cleavage is very close to the N- or C-terminal of the original single chain or very close to the newly formed N-terminal of the heavy chain or C-terminal of the light chain. Of these four possibilities, one is eliminated because, as noted above, the light chain of type B and its parent single chain NT have identical amino terminal sequences. Since the light chain was isolated from a trypsinized and hence fully activated NT, the molecular events leading to activation cannot involve proteolysis at or very close to the N-terminus of the intact type B NT.

Another issue is the question of whether or not endogenous protease and trypsin nick a NT at the same site. Sequence homology between the heavy chains of type A and B suggest cleavage at a common final site because (1) the heavy chain of type A was derived from a preparation that, like most

preparations of type A (17), was endogenously nicked into dichain structure, and (ii) the heavy chain of type B was derived by completing the nicking, with trypsin, of the NT that was isolated in the form essentially not nicked by the endogenous protease(s).

Thus, sequence studies on two widely separated regions of botulinum neurotoxins reveal extensive homologies between pharmacologically similar NT types that are antigenically distinct. The data reported here comprise a very small percentage of the total structures of these relatively large proteins. Nonetheless, this represents the first definitive information on long-held hypotheses and conjectures with respect to alignment of heavy and light chains, homologies and heterologies between serotypes, and molecular events mediating activation and nicking.

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FOOTNOTE

³Abbreviations: NT, neurotoxin. HPLC, high performance liquid chromatography.

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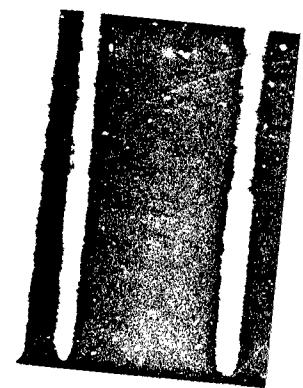
Fig. 1: SDS polyacrylamide electrophoresis of the various purified preparations. In all cases, samples were reduced with mercaptoethanol prior to running on the gel. Left to right: Type E; type B; trypsinized (nicked) type B; purified heavy chain from nicked type B; purified light chain from nicked type B. It should be noted that the gel with type E shown in Figure 1 was not done in the same electrophoresis run as the others, and conclusions with respect to relative molecular weights cannot be drawn from these gels.

Fig. 2: HPLC chromatograms of PTH derivatives from sequencer runs. Panel a, cycle 1 from single chain type E NT. Panel b, cycle 1 from single chain type B NT. (The unlabeled major peak is an internal standard.) Panel c, cycle 1 from the light chain of nicked type B NT. Panel d, cycle 1 from the heavy chain of nicked type B NT.

Fig. 3: Amino terminal sequences of single chain type B NT, of the light and heavy chains from nicked type B NT, and of single chain type E NT. The letter "X" indicates an unidentified residue.

Fig. 4: Part a, a comparison of the amino terminal sequences of the light chain from type A NT (13) and single chain types B and E NT; Part b, a comparison of the amino terminal sequences on the heavy chains from types A (13) and B NT. Within each of the two comparisons, common residues are shown in boldface.

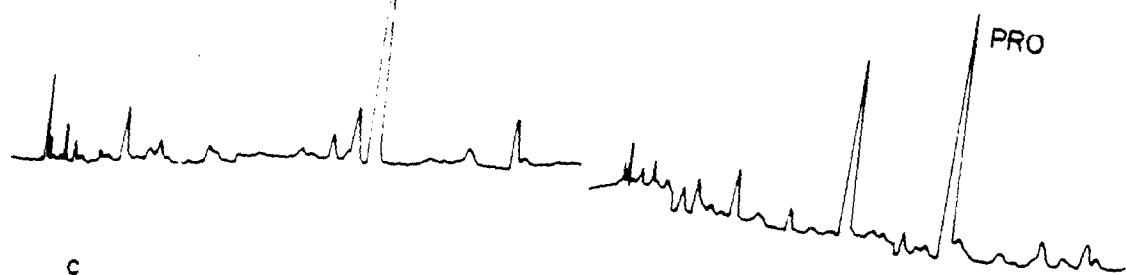




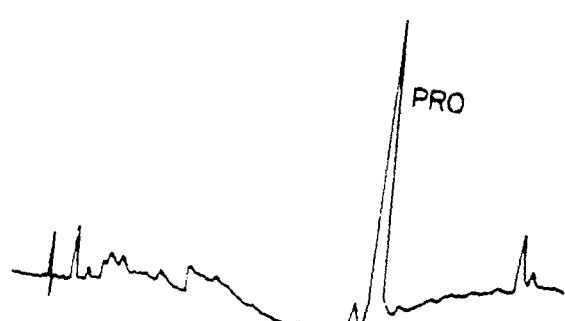
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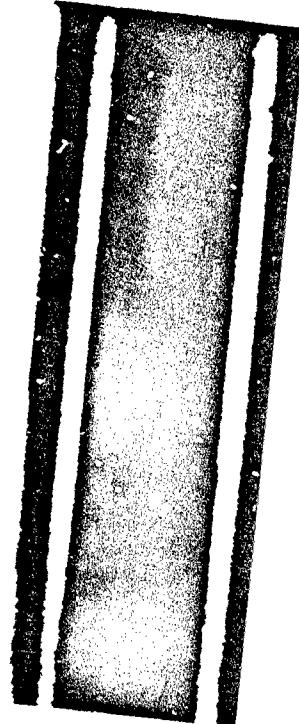
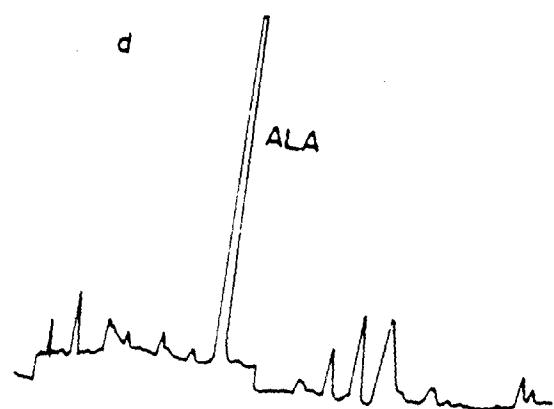
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c



d



TYPE B, SINGLE CHAIN NT :	PRO. VAL. THR. ILE. ASN. ASN. PHE. ASN. TYR. ASN. ASP. PRO. PRO. ILE. ASP. ASN
TYPE B, LIGHT CHAIN :	PRO. VAL. THR. ILE. ASN. ASN. PHE. ASN. TYR. ASN. ASP. PRO. PRO. ILE. ASP. ASN. ASN
TYPE B, HEAVY CHAIN :	ALA. PRO. GLY. ILE. CYS. ILE. ASP. VAL. ASP. ASN. GLU. ASP. LEU. PHE. PHE. ILE. ALA. ASP
TYPE E, SINGLE CHAIN NT :	PRO. LYS. ILE. ASN. SER. PHE. ASN. TYR. ASN. ASP. X . VAL. ASN

a

BOTULINUM A, LIGHT CHAIN:
1 PRO.PHE.VAL.ASN.LYS.GLN.PHE.ASN.TYR.LYS.ASP.PRO.VAL.ASN.GLY.VAL.ASP
5
10
15
BOTULINUM B, SINGLE CHAIN NT: PRO.VAL.THR.ILE.ASN.ASN.PHE.ASN.TYR.ASP.PRO.ILE.ASP.ASN
BOTULINUM E, SINGLE CHAIN NT: PRO....LYS.ILE.ASN.SER.PHE.ASN.TYR.ASP...X...VAL.ASN

b

BOTULINUM A, HEAVY CHAIN:
1 ALA.LEU.ASN.ASP.LEU.CYS.ILE.LYS.VAL.ASN.ASN.ILE.ASP.LEU.LYS.PHE
5
10
15
BOTULINUM B, HEAVY CHAIN:
ALA....PRO.GLY.ILE.CYS.ILE.ASP.VAL.ASP.ASN.GLU.ASP.LEU.PHE.ILE.ALA.ASP